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Overexpression of Corticotropin Releasing Factor in the Central Nucleus of the Amygdala Advances Puberty and Disrupts Reproductive Cycles in Female Rats

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Prolonged exposure to environmental stress activates the hypothalamic-pituitary-adrenal (HPA) axis and generally disrupts the hypothalamic-pituitary-gonadal axis. Because CRF expression in the central nucleus of the amygdala (CeA) is a key modulator in adaptation to chronic stress, and central administration of CRF inhibits the hypothalamic GnRH pulse generator, we tested the hypothesis that overexpression of CRF in the CeA of female rats alters anxiety behavior, dysregulates the HPA axis response to stress, changes pubertal timing, and disrupts reproduction. We used a lentiviral vector to increase CRF expression site specifically in the CeA of preweaning (postnatal day 12) female rats. Overexpression of CRF in the CeA increased anxiety-like behavior in peripubertal rats shown by a reduction in time spent in the open arms of the elevated plus maze and a decrease in social interaction. Paradoxically, puberty onset was advanced but followed by irregular estrous cyclicity and an absence of spontaneous preovulatory LH surges associated with proestrous vaginal cytology in rats overexpressing CRF. Despite the absence of change in basal corticosterone secretion or induced by stress (lipopolysaccharide or restraint), overexpression of CRF in the CeA significantly decreased lipopolysaccharide, but not restraint, stress-induced suppression of pulsatile LH secretion in postpubertal ovariectomized rats, indicating a differential stress responsivity of the GnRH pulse generator to immunological stress and a potential adaptation of the HPA axis to chronic activation of amygdaloid CRF. These data suggest that the expression profile of this key limbic brain CRF system might contribute to the complex neural mechanisms underlying the increasing incidence of early onset of puberty on the one hand and infertility on the other attributed to chronic stress in modern human society. (*Endocrinology* 155: 3934–3944, 2014)

The timing of puberty in mammals, including man, is controlled by a multiplicity of complex interactions between genetic and environmental factors, with the latter providing fine tuning to maximize reproductive potential to fit the prevailing or predicted environment. There is unequivocal evidence that chronic stress suppresses the activity of the

hypothalamo-pituitary-gonadal (HPG) axis and delays puberty. However, less well recognized is the advancement of puberty by environmental factors, such as childhood physical and sexual abuse (1, 2) or familial stress, such as absence of the father or parental conflict (3), with striking parallels in animal models of weak parent-offspring bonding (4).

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Abbreviations: BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CRF-R1, CRF receptor type 1; EPM, elevated plus maze; GFP, green fluorescent protein; HPA, hypothalamo-pituitary-adrenocortical; HPG, hypothalamo-pituitary-gonadal; LPS, lipopolysaccharide; LV, lentiviral vector; MeA, medial amygdala; mPOA, medial preoptic area; pnd, postnatal day; PVN, paraventricular nucleus.

CRF is heterogeneously distributed throughout the brain, but highly concentrated in the paraventricular nucleus (PVN) of the hypothalamus, the core regulatory component of the hypothalamo-pituitary-adrenocortical (HPA) axis, and portions of the extended amygdala, including the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA). Intracerebroventricular administration of CRF or CRF antagonist delays or advances puberty, respectively (5), suggesting CRF regulation of puberty. We have shown a decline in CRF and CRF receptor type 1 (CRF-R1) and CRF-R2 expression in the PVN across the pubertal transition in female rats (5). However, it has been suggested that the PVN is not crucial for stress-induced modulation of the HPG axis by PVN lesion studies (6). Although the site and mechanism of action of the endogenous CRF tone, which plays a critical role in the timing of puberty, remains elusive, an obvious candidate is the CeA because it contains a major CRF neuronal population forming an important component of the limbic brain stress-response network, and because CRF expression in the CeA is important in adaptation to chronic stress, during which CRF is upregulated (7, 8). The importance of the amygdala in the control of adult reproductive function is widely recognized, with different subnuclei subserving stimulatory and/or inhibitory effects on gonadotropic hormone secretion (9, 10). Stimulation and ablation studies in prepubertal rats have revealed a critical role for the amygdala in the timing of puberty, with lesions of the medial amygdala (MeA) advancing or delaying puberty depending on the developmental age in which the manipulation occurred (11), whereas stimulation delayed puberty (12). Although similar studies have not been carried out for the CeA, this subnucleus plays a critical role in stress-induced suppression of the GnRH pulse generator (13). The use of viral vectors to modulate site-specific CRF expression has enhanced understanding of the role of CRF in restricted brain nuclei in mediating stress and reproductive-related behaviors (14). Overexpression of CRF in the CeA increases anxiety, disrupts estrous cyclicity, and reduces GnRH expression in the medial preoptic area (mPOA) in adult rats, strongly suggesting that CRF within the CeA negatively impacts reproductive physiology (14).

Puberty is a time of heightened stress responsivity with earlier onset associated with raised cortisol reactivity (15). Although there is clear evidence that the amygdala is involved in facilitating HPA axis activation and regulating the timing of puberty, at least in rodents (11, 12), its precise role in stress-related change in pubertal timing and reproductive cyclicity remain to be fully established. We hypothesize that overexpression of CRF in the CeA in female rats, which mimics chronic stress exposure, would

increase anxiety behavior and the HPA response to stress and delay puberty and disrupt reproduction. To test this, we used a lentiviral vector (LV) to site-specifically express CRF in the CeA of female rats before weaning to examine the effects of continuous CRF overexpression in this limbic structure on anxiety behavior, the timing of puberty, estrous cycles, and the preovulatory LH surge. We also determined the response of the GnRH pulse generator, a critical regulator of puberty (16–21), to psychological (restraint) and immunological (lipopolysaccharide [LPS]) stressors.

Materials and Methods

Production of recombinant LVs

The cDNA encoding rat CRF was amplified (5'-CCGCTC-GAGCCACCATGCGGCTGCGGCTGCTGGT-3' [*Xho*I] and 5'-CGCAGATCTTCATTTCCCGATAATCTCC-3' [*Bgl*II]) from rat brain cDNA using Phusion high-fidelity DNA polymerase (New England BioLabs) and cloned into compatible restriction sites of pRRL.SIN.CPPT.CMV.IRES-GFP.WPRE (LV-CRF). An LV-expressing green fluorescent protein (GFP) (pRRL.SIN.CPPT.CMV.GFP.WPRE) (LV-GFP) was used as control (Addgene). LVs were propagated in Stbl3-competent cells and plasmid constructs were purified using PureLink HiPure Plasmid Filter Maxiprep kit (Invitrogen). Viruses were generated by transient transfection of the transfer vector together with 3 packaging plasmids (pMDLg/pRRE, pRSV-Rev, and PMD2.G; Addgene) into HEK293T cells by the calcium phosphate method (22). Virus purification, concentration, and titration were performed as previously described (23). All viruses in the present study had a titer of $>3.5 \times 10^9$ IU/mL.

Animals, housing, puberty onset, and estrous cycles

Sprague-Dawley rats (Charles River) were housed under controlled conditions (12-hour light, 12-hour dark cycle, lights on 7:00 AM; temperature $22^\circ\text{C} \pm 2^\circ\text{C}$) and supplied with food and water ad libitum. Litters were reduced to 10 to 12 pups on postnatal day (pnd) 1 (birth, day 0). Litters were weaned on pnd 21 and housed in groups of 3 to 4 per cage, provided with food and water ad libitum, and weighed every 3 days. They were also monitored daily for vaginal opening and first vaginal estrus (markers of puberty onset) from pnd 28. Once vaginal opening occurred, vaginal smears were taken daily for 2 to 3 consecutive weeks to detect the stage of the estrous cycle. Normal estrous cyclicity was defined as having at least 2 consecutive normal cycles, which lasts for 4 to 5 days with 1 to 2 days of estrus. Cycle length, with 3 stages observed in the correct order, was determined by the number of days between each occurrence of estrus. After removal of the ovaries, they were wax-embedded and sectioned ($4\ \mu\text{m}$), and the number of corpora lutea in each 10th section was averaged. All procedures were conducted in accordance with the United Kingdom Home Office Regulations.

Surgical procedures

Rats were anesthetized with a combination of ketamine (Vetalar, 100 mg/kg, ip; Pfizer) and xylazine (Rompun, 10 mg/kg, ip; Bayer) on pnd 12 and placed in a Kopf stereotaxic frame, and holes were drilled bilaterally in the skull at a location above the CeA after a midline incision was made in the scalp. The coordinates 3.2 mm lateral, 2.3 mm posterior to bregma, and 6.6 mm below the surface of the dura were used to target the CeA (24). A glass pipette connected to a tip of a 5- μ L Hamilton microsyringe preloaded with virus was lowered into the target site over a 1-minute period, and another minute elapsed before the injection was initiated. Each animal was infused bilaterally with 1 μ L of either control (LV-GFP; $n = 26$) or CRF-overexpressing (LV-CRF; $n = 25$) virus at a rate of 100 nL/min. The pipette was left in place for 10 minutes after the injection and then slowly removed over 2 minutes. The scalp was closed with suture.

Ten days after puberty, the rats were implanted with 2 cardiac catheters to enable blood sampling for LH and corticosterone measurement or infusion of drugs (17). The catheters were exteriorized at the back of the head and secured to a cranial attachment (17); the rats were fitted with a 30-cm-long metal spring tether (Instec Laboratories Inc), the distal end of which was attached to a fluid swivel (Instec Laboratories), allowing freedom of movement of the rat. After iv catheterization, rats were housed individually, and blood sampling commenced about 2 days later.

Effects of overexpression of amygdala CRF on anxiety

Two days before the first behavioral test was initiated, the rotarod test was used to evaluate motor function (25). The elevated plus maze (EPM) and social interaction tests were performed as described previously (26) to assess anxiety. Ten days after microinjection of the lentivirus into the CeA, the EPM tests were performed at 5- to 6-day intervals until approximately 5 days after puberty. Social interaction was tested only once on pnd 30. Animals were habituated twice (once per day, 2 days before the day of testing) to the behavioral room for 15 minutes and then to the social interaction arena for 5 minutes on each occasion.

Elevated plus maze

The EPM consisted of 4 arms: 2 facing open arms (51×10 cm) and 2 closed arms (51×10 cm, with a wall height of 41 cm), and was elevated 72 cm above the floor. Testing was performed under bright white light. Rats were transferred to the testing room between 9:00 and 10:00 AM and immediately placed in the center of the maze facing the open arm, and the time spent in open and closed arms was recorded for 5 minutes.

Social interaction

The time spent on the behavioral events sniffing, chasing, following, grooming, and mounting were recorded over a period of 15 minutes by an observer blind to the treatment. The tests were carried out between 9:00 and 11:00 AM with the pairs of unfamiliar rats, weight-matched and from the same treatment, placed simultaneously in the $50 \times 50 \times 50$ -cm arena. The total social interaction time was calculated by the sum of the time spent in the active social behaviors noted above.

Effects of overexpression of amygdala CRF on LH surge and response to stress

LH surge

Once the proestrous vaginal cytology was detected by the daily smears carried out in the morning, rats were attached via 1 of the 2 cardiac catheters to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of small blood samples (25 μ L) without disturbing the animals. Once connected, animals were left undisturbed for 1 hour before sampling commenced. Blood sampling commenced at noon, and samples were collected every 30 minutes for 8 hours for LH surge analysis. After removal of each 25- μ L blood sample, an equal volume of heparinized saline (50 U/mL normal saline; CP Pharmaceuticals) was automatically infused into the animal to maintain patency of the catheter and blood volume.

LH pulses and response to stressors

After blood sample collection for detection of the spontaneous LH surge, rats were subsequently ovariectomized (27) to facilitate LH pulse detection, and after a period of about 7 days, blood samples were collected every 5 minutes for 6 hours for the measurement of LH. After 2 hours of controlled blood sampling for baseline LH pulses, the animals were exposed to stressors. For restraint stress, the animals were placed in a restraint device for 60 minutes and automated blood sampling continued uninterrupted during restraint and the 3-hour postrestraint period. On a subsequent occasion, separated by at least 4 days, the animals were exposed to an immunological stressor: 2 hours into the 6-hour blood sampling procedure, LPS (20 μ g/kg in 0.2 mL saline; Sigma-Aldrich Ltd) was injected via the second cardiac catheter (25). Manual blood samples (25 μ L) were collected via the second catheter immediately before and 30, 60, 120, and 240 minutes after restraint or LPS stress onset. Blood samples were frozen at -20°C for later assay to determine LH and corticosterone concentrations.

Measurement of LH and corticosterone concentrations

A double-antibody RIA supplied by the National Institute of Diabetes and Digestive and Kidney Diseases was used to determine LH concentration in the 25- μ L whole-blood sample. Reference preparation was rLH-RP-3. The sensitivity of the assay was 0.093 ng/mL. The intra-assay variation was 5.3%, and the interassay variation was 7.5%. A double-antibody RIA (ImmuChem; MP Biomedicals) was used to determine the corticosterone concentration in the plasma samples (10 μ L) following the manufacturer's protocol. The intra-assay variation was 8.2%, and all samples were analyzed on single determinations for corticosterone.

Immunocytochemistry and verification of GFP expression site

Animals were deeply anesthetized with sodium pentobarbital (Rhone Merieux Ltd) and transcardially perfused with 4% paraformaldehyde for 15 minutes. The brains were removed, placed in postfix solution and then transferred to 30% sucrose. Brains were stored at -80°C and coronally sectioned (30 μ m) at a later date. To evaluate the LV-CRF or LV-GFP injection sites, every sixth free-floating section throughout the CeA region was

mounted after sectioning and assessed directly under a fluorescent microscope for GFP visualization. Rats that did not show GFP at the target injection site were excluded from data analysis.

Every sixth free-floating section throughout the CeA region was used for CRF immunostaining. Sections were incubated in 1:2500 rabbit anti-CRF primary antibody (Peninsula Laboratories, Inc) containing 1% normal goat serum at room temperature for 24 hours, after which they were processed with a rhodamine red TM-X goat antirabbit IgG (H+L) (Life Technologies Ltd) for 2 hours. The sections were mounted and counterstained with Prolong Gold antifade reagent (Life Technologies). Omission of the CRF primary antibody resulted in the absence of specific staining. Several brains from each experimental group were processed in the same batch to control for interbatch variability. Immunopositive staining CRF cells were quantified at $\times 40$ magnification on an AxioVision microscope image system (Zeiss). All analyses were performed blinded to experimental group by 2 investigators. The boundaries of the CeA were determined by comparing the rat brain atlas (24) with neuroanatomical landmarks. Sections used for the CeA analysis were taken from the region corresponding to bregma -2.12 to -3.14 mm. The number of neurons expressing CRF immunoreactivity was determined bilaterally in 4 sections from each rat, and the average value used to calculate the group mean.

Statistical analysis

Comparisons between LV-GFP and LV-CRF groups with respect to body weight, day of vaginal opening and first estrus, cycle length and time spent in each phase, number of corpora lutea, and behavioral parameters were made using a one-way ANOVA followed by Dunnett's test. The percentage of normal estrous cycles was compared using χ^2 test. Comparison of CRF neuron number and the presence of LH surges between groups were analyzed using the Mann-Whitney rank sum test. To evaluate LH surges, area under the curve of the LH profile was calculated during the 8-hour sampling period and compared using ANOVA. The algorithm ULTRA was used to establish the detection of LH pulses (28). Two intra-assay coefficients of variation of the assay were used as the reference threshold for the pulse detection. The inhibitory effects of restraint or LPS stress on LH pulses in the treatment and control groups were calculated by comparing the mean LH pulse interval before stress with the first prolonged interval after stress onset. For the restraint stress, the first prolonged interval corresponded to the first LH pulse interval after stress onset. In the case of LPS stress, the first prolonged LH pulse interval was delayed by approximately 25 minutes after LPS injection. The poststress recovery period was defined as the remaining observation period after the first prolonged LH pulse interval. Statistical significance of LH pulse frequencies and amplitudes and mean plasma levels of corticosterone at different time points relative to stress between treatment and control rats was tested using ANOVA. $P < .05$ was considered statistically significant.

Results

Site-specific overexpression of CRF

Seventeen of 26 rats injected with LV-CRF and 15 of 25 rats treated with LV-GFP virus verified with correct bi-

lateral detection of GFP in the CeA were included in the data analysis. Infusion of LV-CRF virus into the CeA resulted in overexpression of CRF to the CeA (Figure 1, C and D) compared with control animals injected with LV-GFP (Figure 1, A and B). The CRF overexpression was also limited to the CeA (Figure 1C). The number of CRF neurons was significantly higher in the rats treated with LV-CRF compared with controls (Figure 1F; $P < .05$).

Puberty and estrous cyclicity

Bilateral administration of LV-CRF virus into the CeA on pnd 12 significantly advanced puberty onset compared with controls (vaginal opening: 36.08 ± 0.69 vs 38.44 ± 0.93 days, respectively; Figure 2A; $P < .05$; first estrus: 36.30 ± 0.61 vs 38.62 ± 0.93 days, respectively, mean \pm SEM; $P < .05$). However, there was no significant difference in body weight gain (Figure 2B). Treatment with LV-CRF disrupted estrous cyclicity in the postpubertal period (Figure 2C; $P < .05$). The majority (76%) of LV-GFP control rats showed normal 4- to 5-day estrous cycles after puberty, whereas only 32% of the LV-CRF-treated animals showed normal cyclicity (Figure 2C; $P < .05$). Representative examples of estrous cycles from each group are illustrated in Figure 2D. Cycle length was also prolonged (Figure 2E; $P < .05$), and a predominance of estrous phase evident (Figure 2F; $P < .05$) in LV-CRF-treated animals. The number of corpora lutea was significantly less in the LV-CRF-compared with LV-GFP-treated animals (number per ovarian section: 3.91 ± 0.83 vs 10.38 ± 0.61 , respectively; mean \pm SEM; $n = 8-9$ per group; $P < .05$).

Behavioral effect of CeA CRF overexpression

Elevated plus maze

The EPM was performed once every 5 to 6 days starting 10 days after microinjection of LV-CRF or LV-GFP and ended about 5 days after puberty. In the control animals, the percentage of time spent in the open arms decreased with approaching puberty, reaching a nadir at 9 to 5 days before vaginal opening and followed by a marked increase after puberty (1–5 days) (Figure 3; $P < .05$). In animals overexpressing CRF, there was a more marked and significant decrease in the percentage of time spent in the open arms with puberty development compared with controls (Figure 3; $P < .05$). Although the percentage of time spent in the open arms increased after puberty, this was significantly attenuated by LV-CRF (Figure 3; $P < .05$). Rotarod analysis revealed no difference between treatment groups in the time the rats stayed on the rotating rod (data not shown), indicating an absence of impaired motor function.

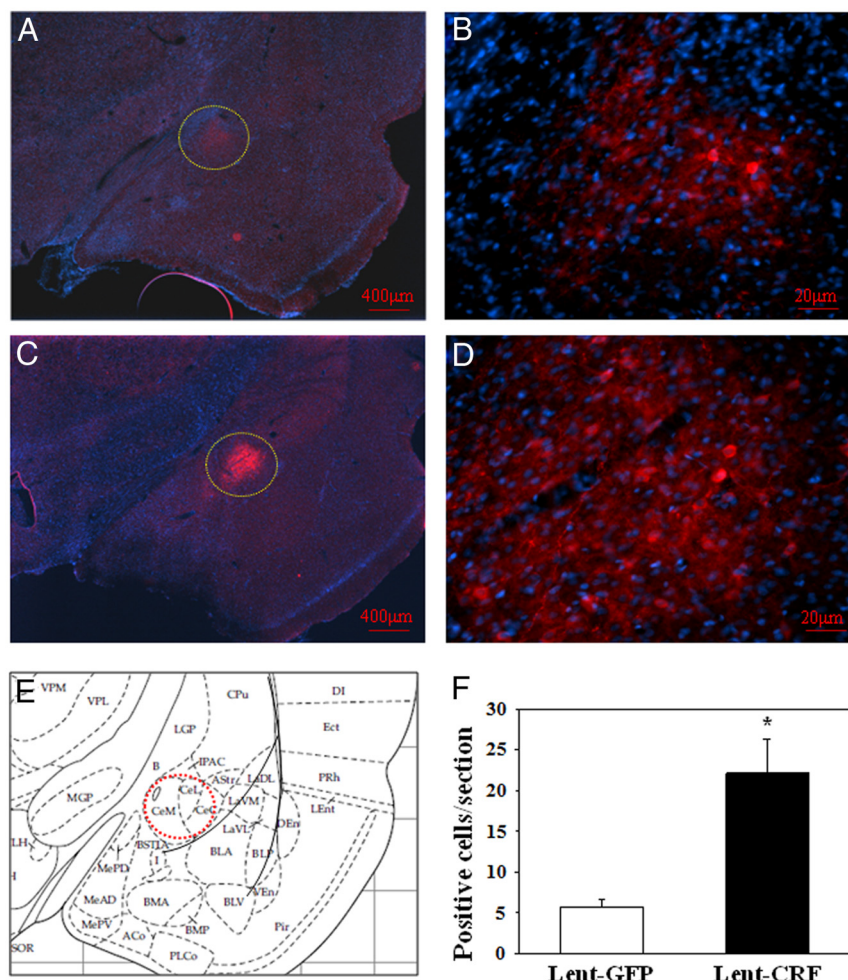


Figure 1. Lentivirus expressing CRF (LV-CRF) microinjection into the CeA on pnd 12 significantly increased CRF protein production site-specifically. A, Representative section used to quantify the number of CRF-immunopositive staining neurons in the CeA from a rat injected with the control virus, expressing GFP (LV-GFP). B, Enlargement of the CeA region corresponding to the control virus injection site illustrated in A. C, Representative section showing the effects of LV-CRF injection into the CeA on the number of positive labeled CRF neurons. D, Enlargement of the CeA region corresponding to the LV-CRF injection site illustrated in C. E, Schematic representation of site of injection adapted from Paxinos and Watson (24) rat brain atlas. F, Summary of number of positive labeled CRF cells per section in the CeA of LV-GFP- and LV-CRF-treated rats determined by immunocytochemistry. *, $P < .05$ vs LV-GFP control. Results represent mean \pm SEM ($n = 15$ – 17 per group).

Social interaction

The social interaction testing was performed on pnd 30. There was a marked impact of CRF overexpression in the CeA with a significant decrease in the cumulative mean time spent engaged in social interaction behavior (including sniffing, chasing, following, grooming, and mounting) between the LV-GFP control (120.25 ± 35.13 seconds) and LV-CRF (30.14 ± 3.41 seconds) treatment groups ($n = 15$ – 17 per group; $P < .05$).

Effects of overexpression of amygdala CRF on LH surge and response to stress

LH surge

Low basal levels of LH were seen between noon and 2:00 PM on the day of proestrus in all experimental rats.

Seven of 9 rats from the control group expressed the LH surge on proestrus, typically starting at 3:00 PM with high LH levels maintained until 6:00 PM (Figure 4A). However, all 6 rats from the LV-CRF group failed to show an LH surge and maintained low levels of LH throughout the 8-hour sampling period on the day of proestrus (Figure 4B). The area under the curve of the LH profile calculated for the 8-hour sampling period quantified the LH surge (2171.97 ± 511.16 vs 937.96 ± 240.91 ng/mL/h; LV-GFP vs LV-CRF group, respectively; $P < .05$).

Pulsatile LH secretion and response to restraint stress

Regular pulsatile LH secretion was observed during the 2-hour baseline blood sampling period, with no significant difference in LH pulse interval between the LV-GFP control ($n = 12$) and LV-CRF treatment ($n = 11$) groups (Figure 5, A–C). Restraint stress induced an immediate suppression of pulsatile LH secretion, with no significant difference between treatment groups (Figure 5, A–C). There was also no significant difference in LH pulse amplitudes before (4.17 ± 0.58 vs 4.20 ± 0.59 ng/mL) and after (4.32 ± 0.62 vs 4.20 ± 0.45 ng/mL; mean \pm SEM) restraint stress between the LV-CRF and LV-GFP groups, respectively.

Pulsatile LH secretion and response to LPS stress

Similarly, there were no significant differences in LH pulse interval before immunological stress onset between the LV-GFP control ($n = 12$) and LV-CRF treatment ($n = 10$) groups (Figure 5, E–G). A prolongation of LH interpulse interval was detected in all animals treated with LPS (Figure 5, E–G). However, the animals with LV-CRF showed a significantly shorter prolongation of LH pulse interval compared with the control group in response to LPS (Figure 5, E–G; $P < .05$). The LH pulse interval in the postrecovery period was comparable between groups (Figure 5, E–G). There was no difference in LH pulse amplitude before LPS stress (3.94 ± 0.56 vs 3.81 ± 0.46

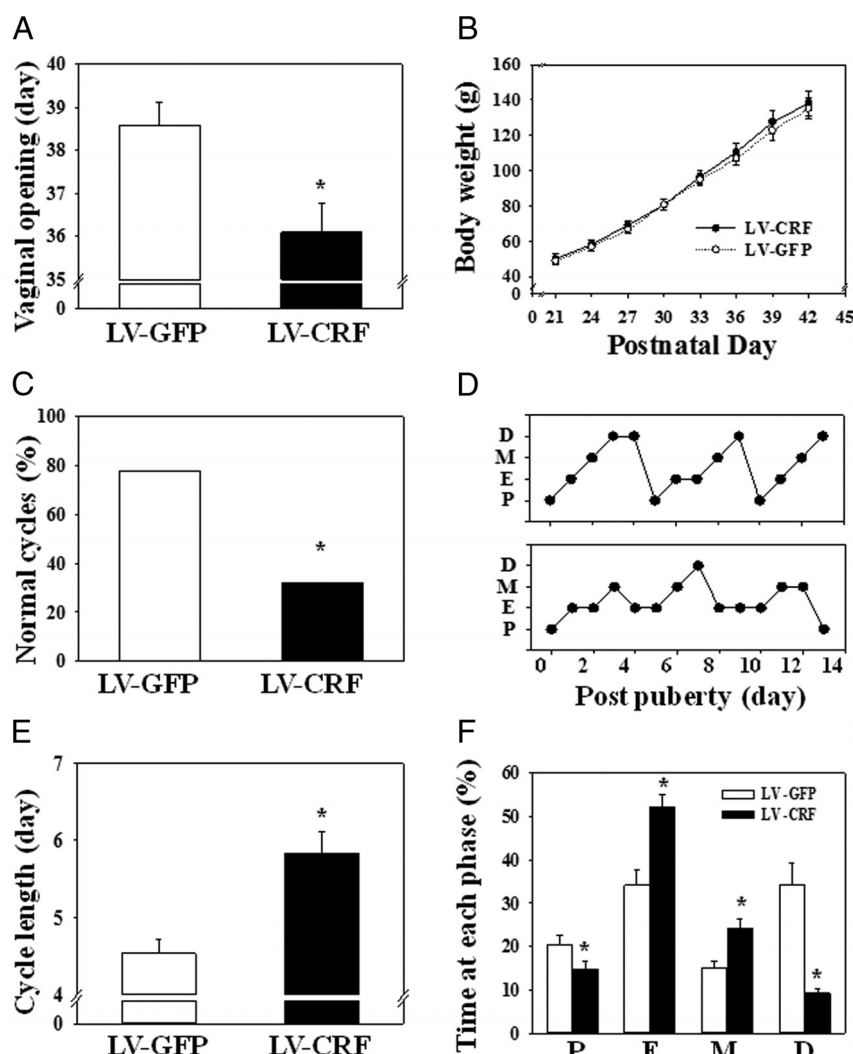


Figure 2. Lentivirus expressing CRF (LV-CRF) microinjection into the CeA on pnd 12 advanced puberty and disrupted estrous cyclicity in female rats. Panel A, Data are presented as day of vaginal opening (puberty) in rats injected with LV-CRF compared with control virus expressing GFP (LV-GFP). Panel B, There was no significant difference in body weight gain between LV-CRF and LV-GFP. Panel C, The percentage of normal estrous cycles was significantly decreased immediately after puberty in LV-CRF-treated compared with LV-GFP control rats. Panel D, Representative examples of estrous cyclicity are illustrated: the upper panel from a normal control rat and lower panel from a rat injected with LV-CRF. Panel E, Length of estrous cycles was significantly increased by LV-CRF-treated compared with LV-GFP. Panel F, The effect of treatment on the percentage of time spent within each estrous cycle phase is shown; LV-CRF primarily increased estrus and decreased diestrus. *, $P < .05$ vs LV-GFP control. Results represent mean \pm SEM ($n = 15$ – 17 per group). Abbreviations: D, diestrus; E, estrus; M, metestrus; P, proestrus.

ng/mL) or during the recovery period (4.01 ± 0.61 vs 4.25 ± 0.63 ng/mL; mean \pm SEM) between the LV-CRF and LV-GFP groups respectively.

Basal and stress-induced corticosterone release

To evaluate whether changes in the levels of CRF in the CeA modified HPA axis activity, corticosterone levels under basal conditions and in response to restraint or LPS stress were determined. Rats had low basal plasma levels of corticosterone before restraint or LPS stress and no significant difference between groups (Figure 5, D and H).

Both restraint and LPS increased plasma levels of corticosterone (Figure 5, D and H) as described previously (29). However, there was no significant difference in the corticosterone response to either stressor between the CRF overexpression and control groups.

Discussion

The present study demonstrates increased anxiety-like behavior and advancement of puberty onset followed by irregular estrous cycles with CRF overexpression in the CeA. These results demonstrate for the first time a novel role for the classical stress neuropeptide CRF within the CeA on the timing of puberty. The CeA, which contains one of the largest populations of CRF neurons outside of the hypothalamus, is a key integrator of autonomic, behavioral, and neuroendocrine responses to stress (30). We have previously shown that an endogenous CRF tone plays a role in modulating the timing of puberty (5). Although we observed a reduction in CRF and CRF-R1/R2 expression in the PVN across the pubertal transition in female rats (5), the PVN may not be crucial for control of pulsatile LH secretion (6), which is critical for puberty onset (16, 19). In the amygdala, CRF receptors demonstrate age- and sex-specific changes in binding across puberty (31). Although CRF receptor binding was lowest overall in the CeA compared with other amygdala

subnuclei, and CRF-R1 was unchanged, CRF-R2 binding increased more in male than in female rats across puberty (31). Exposure to peripubertal stress, however, enhanced CRF-R1 but not CRF-R2 expression in the CeA, which was associated with increased anxiety (32). Whether these changes in the CeA are linked to pubertal timing was not studied.

In the Japanese quail, genetic selection for high or low HPA axis stress responsivity delays or advances puberty, respectively (33). Mice overexpressing CRF demonstrate

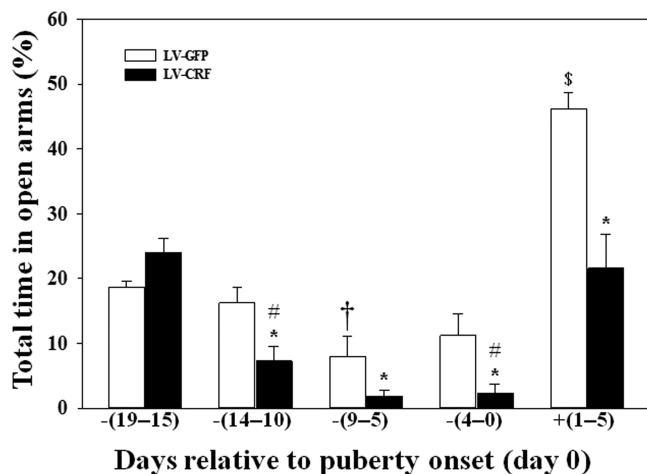


Figure 3. Effects of overexpression of CRF in the CeA on anxiety-like behavior evaluated using the EPM across pubertal development in the female rat. Lentivirus expressing CRF (LV-CRF) injected animals spent significantly less time in the open arms of the EPM (more anxious) compared with control virus (LV-GFP) injected rats. Data are aligned to the day of puberty (vaginal opening, day 0). *, $P < .05$ vs controls at same time point; #, $P < .05$ vs -19 to -15 days time point in same treatment group; \$, $P < .05$ vs all other time points in same treatment group; †, $P < .05$ vs control LV-GFP group at -19 to -15 and -14 to -10 days time points in same treatment group ($n = 15$ –17 per group).

raised corticosterone levels, are stress hyperresponsive, and display reduced fertility (34). Juvenile female rats receiving intracerebroventricular infusion of CRF had delayed puberty, although corticosterone levels were unchanged (5). Furthermore, central administration of a nonselective CRF receptor antagonist advanced puberty, indicating an endogenous CRF tone might be a key component of the pubertal timing mechanism (5). CRF and various stressors downregulate hypothalamic mPOA and arcuate nucleus kisspeptin in adult rats (35, 36). Moreover, early life stress, such as neonatal exposure to LPS, which delays puberty, also downregulates kisspeptin, a major gatekeeper of puberty (5), suggesting an action of

CRF on kisspeptin signaling might underlie stress-induced delay of puberty.

Contrary to our expectation, in the present study, puberty was advanced in rats overexpressing CRF in the CeA. There are several situations where stressful environmental conditions are associated with advanced puberty. Girls encountering familial conditions that are unfavorable, such as family stress and poor parent-child relations, have been found to demonstrate early menarche (1–3, 37). Absence of a biological father has been reported to advance puberty in girls (3), and childhood physical and sexual abuse more robustly advances puberty and is accompanied by increased anxiety (1, 2, 37). There is considerable evidence that parental care serves as a mediator for the effects of environmental adversity on development (4). In animal models of weak parent-offspring bonding, such as Meaney's low licking and grooming rats, the offspring are not only more fearful and show increased HPA responses to stress, but have raised CRF expression in the CeA and are reported to have advanced puberty (4). In general, advancement of puberty in high-risk environments where survival is potentially less certain may be an adaptive response, presumably increasing capacity to reproduce, which could have an evolutionary advantage (38).

The advancement of puberty in rats overexpressing CRF in the CeA may result from mechanisms of habituation that reduce the inhibitory influence of daily life stress on the GnRH pulse generator. Indeed, we have shown in the present study an attenuation of LPS stress-induced suppression of LH pulses in these animals. Although CeA lesions do not affect LH pulse frequency under stress-free conditions (13), we have previously shown that LPS stress-induced suppression of LH pulses was blocked by CeA lesions (13). Therefore, overexpression of CRF in the CeA might be expected to negatively affect the GnRH pulse generator. Nevertheless, neither basal corticosterone levels nor the corticosterone response to stress indicated an upregulation of the HPA axis in the CeA CRF overexpression animals, which might support the notion of habituation or adaptation under chronic stress conditions.

In the present study, irregular estrous cyclicity accompanied by lack of LH surges was observed in the postpubertal period of rats overexpressing CRF in the CeA, which is consistent with previous reports showing the involvement of CeA CRF in reproductive dysfunction in adult rats (14). Specifically, continuous overexpression of CRF in the CeA lengthened the estrous cycle or induced acyclic, and significantly decreased GnRH levels in the mPOA (14). In the rhesus monkey, psychosocial stress associated with subordinate status reduces fertility, secondary to an increased incidence of anovulation (39). Rats

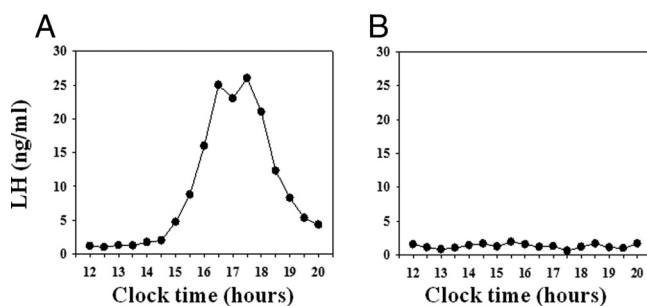


Figure 4. The effects of CRF overexpression in the CeA on the spontaneous preovulatory LH surge. A, Representative example of an LH surge on the day of proestrus in a rat microinjected with control virus expressing GFP (LV-GFP). B, An example showing no preovulatory LH surge from a rat injected with lentivirus expressing CRF (LV-CRF) into the CeA. Blood samples were collected between noon and 8:00 PM on the day of proestrus for the measurement of LH.

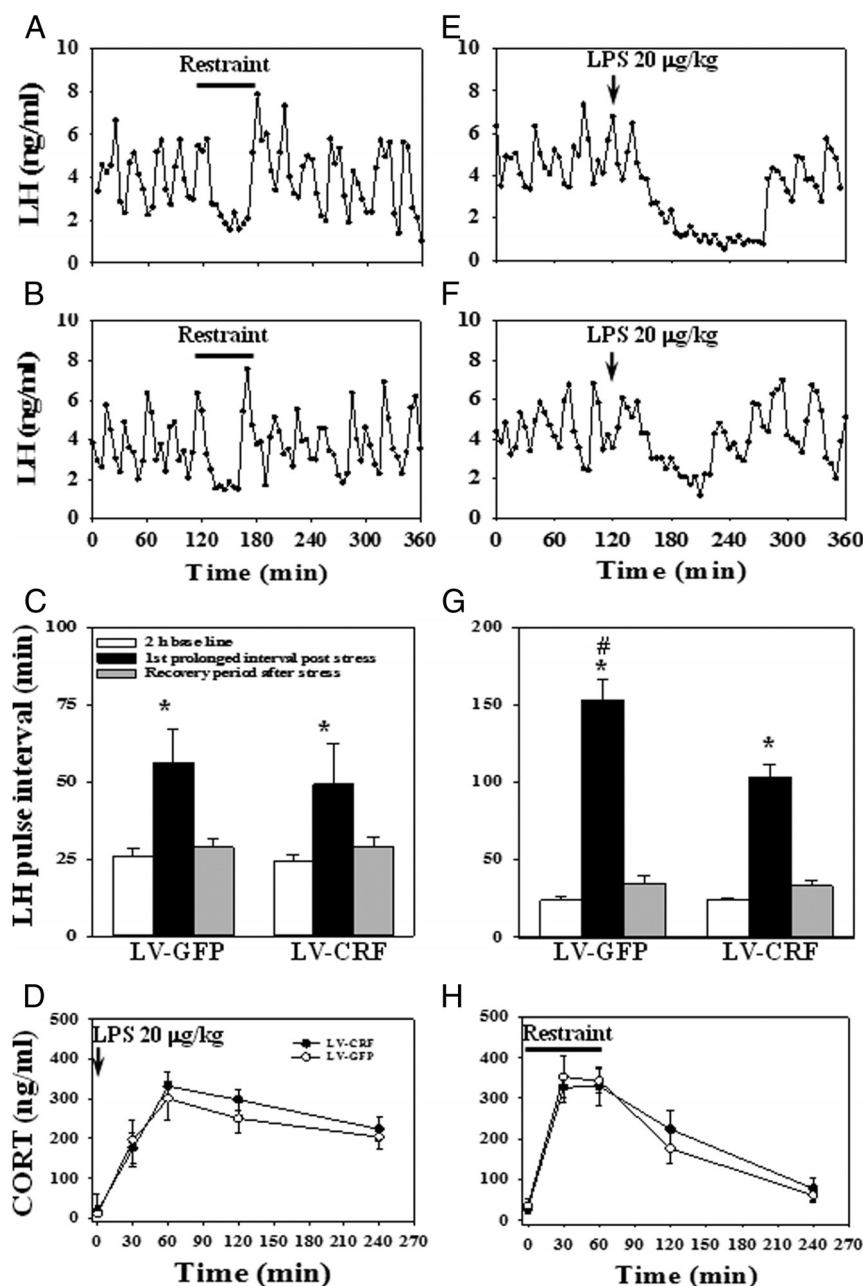


Figure 5. Effect of lentivirus expressing CRF (LV-CRF) microinjected into the CeA prepubertally (pnd 12) on stress-induced suppression of pulsatile LH secretion in rats subsequently ovariectomized postpubertally to facilitate LH pulse detection and stress-induced increase in plasma levels of corticosterone (CORT). Representative examples illustrating the effect of restraint (1 hour) stress on pulsatile LH secretion in control virus expressing GFP (LV-GFP) (A) or lentivirus expressing CRF (LV-CRF) (B) injected animals. Representative examples illustrating the effect of LPS (20 $\mu\text{g/kg}$ iv) stress on pulsatile LH secretion in control virus (LV-GFP) (D) or LV-CRF (E) injected animals. Summary showing that treatment with LV-CRF to overexpress CRF in the CeA attenuated the inhibitory effects of LPS (F) but not restraint (C) stress on pulsatile LH secretion. The increased plasma levels of CORT in response to restraint (1 hour) (D) or LPS (20 $\mu\text{g/kg}$ iv) (H) stress were not significantly different between the LV-CRF and control LV-GFP group. *, $P < .05$ vs prestress control period within the same group; #, $P < .05$ vs LV-CRF group at the same time point. Results represent mean \pm SEM ($n = 10\text{--}12$ per group).

subjected to chronic mild stress show reduced estrous cyclicity (40). Cynomolgus monkeys that exhibit immediate suppression of menstrual cycles and anovulation upon

indirect functional route for amygdaloid influence on kisspeptin and gonadotrophic hormone secretion. We cannot rule out the possibility, however, that the absence of an LH

stress onset, termed stress-sensitive, have greater density of CRF fibers in the CeA compared with stress-resilient conspecifics that continue to cycle normally (41). Therefore, it is suggested that long-term increase of CRF in the CeA may be a causal factor in stress-related reproductive disorders, including functional hypothalamic amenorrhea, and may help to explain the loss of normal reproductive function after early puberty in the present study.

It is well established that the amygdala is involved in the control of ovulation in rodents. Lesioning of the MeA blocked ovulation (42), whereas stimulation advanced the time of the LH surge (43). Stimulation of the basolateral amygdaloid nucleus or CeA blocked or delayed the LH surge, respectively (43). Thus, the amygdala exerts a dual effect on the preovulatory LH surge, the basolateral and CeA being inhibitory and the MeA facilitatory. However, the mechanism by which CRF in the CeA is involved in the control of the LH surge or ovulation, as alluded to in the present study, remains to be elucidated. Although there are extensive projections from the MeA to the GnRH-rich region of the mPOA (44), there are very few (45) or no (46, 47) neuronal projections from the CeA to the GnRH-rich mPOA or kisspeptin-rich anteroventral periventricular nucleus that controls ovulation. Nevertheless, the CeA is known to densely innervate the BNST and locus coeruleus, primarily with CRF-containing neurons (48, 49), which in turn projects to the arcuate nucleus (50, 51) and mPOA (52, 53). Moreover, the dorsolateral part of the anterior BNST, stimulation of which blocks the LH surge (9), is preferentially innervated by the CeA (50) and thus provides an

surge in the CRF-overexpressing group is due to an inadequate estradiol positive feedback signal; the measurement of circulating levels of this steroid is needed in future studies. An additional caveat is the possibility of a delay in the timing of the LH surge beyond 8:00 PM, when we stopped sampling. Nevertheless, reduced ovulatory efficiency is evident with a significant decrease (62%) in the number of corpora lutea and a complete absence in 2 of the 8 animals overexpressing CRF. The present study suggests that the CeA is a key component in the forebrain stress neural circuitry affecting ovulation. Why overexpression of CRF in the CeA promotes early activation of the HPG axis but suppresses its activity in adulthood is a conundrum. The neural mechanisms underlying puberty onset vs maintenance of ovarian cyclicity might be differentially regulated by the CeA and requires further study. However, parallels are seen; for example, obesogenic diets, which advance puberty (17, 54), can also induce irregular estrous cycles in adult rodents (55, 56) (Li, X. F., unpublished observation). Although advancement of puberty after overnutrition is associated with upregulation of hypothalamic kisspeptin (17, 54), the role of kisspeptin in the disruption of ovarian cyclicity after overnutrition has not been fully characterized (56) and the dynamics of kisspeptin signaling remains to be examined in the model of CeA CRF overexpression.

Overexpression of CRF in the CeA is thought to recapitulate many of the effects of chronic stress. Lesioning the CeA attenuates stress-induced anxiety behavior and corticosterone release (57, 58). Overexpression of CRF in the CeA increases HPA axis reactivity, enhances the ACTH and corticosterone response to a dexamethasone/CRF test, increases the expression of both CRF and AVP in PVN, and reduces glucocorticoid negative feedback (14, 59). Resistance to glucocorticoid feedback inhibition is present in many chronic stress conditions, including anxiety disorders and functional hypothalamic amenorrhea, due to decreased central glucocorticoid receptor function (60). However, other studies have failed to observe changes in the HPA axis after CeA CRF overexpression (61). In the present study, there was no evidence of upregulation of the HPA axis both in terms of basal or stress-induced corticosterone release. An absence of altered basal levels of corticosterone is in agreement with previous publications using site-specific overexpression of CRF in the CeA (14, 30, 61, 62). Moreover, there is a significant increase in basal corticosterone release in the CeA-CRF knockdown mouse (62). The corticosterone response to restraint stress was not modified by prolonged CRF overexpression in the CeA of mice (61). Chronic stress, which is known to upregulate CeA CRF, attenuates the stress response through habituation of the HPA axis (60). The

increase in AVP in PVN is a well-established consequence of chronic stress (63). However, CRF expression in the PVN has been shown to remain at basal levels under these conditions (63). Although tonic elevation of plasma corticosterone due to chronic stress may inhibit the HPA axis via negative feedback mechanism, the HPA axis retains responsiveness to new stressors (63). The peripubertal rats used in the present study may have greater neural plasticity and developmental changes induced by the early-life onset of CRF overexpression (64) and therefore did not show a significant change in the corticosterone response to LPS or restraint stress. This may be crucial for the individual's survival and health, thereby limiting the stress response and preventing pathologies associated with excess CRF levels in CeA such as in chronic stress condition.

We demonstrated that overexpression of CRF in the CeA decreased open arm exploration in the EPM and social interaction in the peripubertal rats, which further confirms previous reports suggesting a central role for CRF in this limbic brain region in control of anxiety-like behaviors in adult animals (59). However, Flandreau et al (30), in contrast to the present study, did not show any changes in social interaction after overexpression of CRF in the CeA of adult rats, but they did observe increased anxiety-like behavior in the defensive withdrawal test. Although Regev et al (62) reported that CeA CRF overexpression did not increase basal anxiety-like behavior, they did show augmented behavioral responses to acute stress exposure indicative of enhanced anxiogenic effects in mice. However, it is of note that knockdown of CRF in the CeA resulted in a decrease in anxiety-like behavior as measured using the EPM (62). Although a decrease in anxiety behavior is seen in late adolescent rats (65), the dramatic and sudden nature of the decrease observed immediately post puberty in the present study is novel. The adolescent brain undergoes dramatic developmental change (66) controlled in part by gonadal steroids (67); however, it remains to be established whether they underlie this phenomenon.

In conclusion, overexpression of limbic brain CRF with concomitant enhanced anxiety might provide a novel mechanism for early puberty associated with early-life adversity. Additionally, continued longer-term hyperactivation of CRF in the CeA might contribute to disruption to ovarian cyclicity and ovulation and hence infertility in adulthood.

Acknowledgments

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